

1           **PROCESS FOR PRODUCING LIPOSOME SUSPENSION AND**  
2           **PRODUCT CONTAINING LIPOSOME SUSPENSION PRODUCED**  
3                           **THEREBY**

4           **BACKGROUND OF THE INVENTION**

5           1. Field of the Invention

6                   The present invention generally relates to a process for producing a  
7           liposome suspension and a liposome suspension produced thereby. The present  
8           invention also relates to a process for producing a product containing a liposome  
9           suspension and the product produced thereby, and particularly to a process for  
10          producing a liposome-encapsulated drug and the liposome-encapsulated drug  
11          produced thereby.

12          2. Description of Related Art

13                  Since Dr. Alec D. Bangham disclosed the concept of using liposome as a  
14          delivery vehicle and the technique of efficiently manufacturing liposome in 1960  
15          in England, liposome has become as an important target of research in the field  
16          of drug delivery. Liposomes are most frequently prepared from phospholipids,  
17          but other molecules of similar molecular shape and dimensions having both a  
18          hydrophobic and a hydrophilic moieties can be used.

19                  Liposomes are microscopic vesicles, generally spherically shaped,  
20          formed from one or more lipid walls. The walls are prepared from lipid  
21          molecules, which have the tendency both to form bilayers and to minimize their  
22          surface area. The lipid molecules that make up a liposome have hydrophilic and  
23          lipophilic portions. Upon exposure to water, the lipid molecules form a bilayer  
24          membrane wherein the lipophilic portions of the lipid molecules in each layer are

1 directed to the center of the membrane, and the opposing hydrophilic portions  
2 form the respective inner and outer surfaces of the bilayer membrane. Thus, each  
3 side of the membrane presents a hydrophilic surface while the interior of the  
4 membrane presents a lipophilic medium. The micelle liposome can have a  
5 particle size less than 20 nm. The micelle liposome can be used as a vector to  
6 deliver biologically active material to the body of human or animal in  
7 pharmaceutical manufacturing.

8         Liposomes can be classified into several categories based on their  
9 overall size and the nature of the lamellar structure. The categories include small  
10 unilamellar vesicles (SUV), multilamellar vesicles (MLV), large unilamellar  
11 vesicles (LUV), and oligolamellar vesicles (OLV). The range of the particle size  
12 of MLVs is about 0.4  $\mu\text{m}$  to 1  $\mu\text{m}$ . The range of the particle size of OLVs is about  
13 0.1  $\mu\text{m}$  to 1  $\mu\text{m}$ . The range of the particle size of SUVs is about 20 nm to 100 nm.  
14 The range of the particle size of LUVs is about 1000 nm (R.R.C. New, ed.  
15 Liposomes: a practical approach, 1990, Oxford).

16         Conventional processes for producing liposomes are, for example,  
17 hydration, ultrasonification, dialysis and dilution processes, reverse phase  
18 evaporation (U.S. Pat. No. 4,235,871), surfactant treatment, dehydration-  
19 rehydration or dry-reconstitution, freeze and thaw (U.S. Pat. No. 6,355,267),  
20 pore extrusion, high pressure homogenization and so on (A. D. Bangham, M. M.  
21 Standish, J. C. Watkins, J. Mol. Biol., 13,238-252,1965; R. R. C. New, ed.  
22 Liposomes: a practical approach, 1990, Oxford; G. Gregoriadis, ed. Liposome  
23 Technology, vol. 1, 1993; C. R. C., H. Talsma and D. J. A. Crommelin,  
24 Pharmaceutical Technology, 96-106, 1992). The pore extrusion process and the

1 high pressure homogenization process are used for large scale production of  
2 liposomes and have industrial potential. Based on economic considerations, the  
3 pore extrusion process is a better choice.

4 U.S. Pat. No. 4,737,323 discloses a method for using the pore extrusion  
5 method to produce a liposome suspension. The particle size of the liposome is  
6 uniform and has an average size less than about 0.4  $\mu\text{m}$ . The method comprises  
7 providing a suspension of heterogeneous-size liposomes containing a substantial  
8 portion with sizes greater than 1.0  $\mu\text{m}$ , and passing the suspension under  
9 pressure through an asymmetric ceramic filter whose inner-surface pore size is  
10 greater than the desired average liposome size and no greater than about 1.0  $\mu\text{m}$ .  
11 According to the method, an organic solvent, e.g. chloroform in example 1, is  
12 used to dissolve lipids and then the organic solvent is removed by rotary  
13 evaporation under reduced pressure such that a dried thin film of lipids is formed.  
14 An aqueous medium is added continuously to the dried thin film of lipids, and  
15 the lipids are allowed to swell such that liposomes are formed.

16 Steven Lehigh's U.S. Pat. Nos. 5,004,611 and 5,053,217 disclose a  
17 method of making an aqueous dispersion of liposome that consists of a  
18 biologically active compound. The method consists essentially of mixing a pre-  
19 liposome composition comprising a uniform mixture of: (a) at least one bilayer  
20 forming membrane lipid, and (b) at least one non-aqueous liquid consisting  
21 essentially of a water-miscible organic liquid that is a solvent for the lipid. The  
22 proportion by weight of (a) to (b) is from 40:1 to 1:20.

23 Sufficient water is added to the pre-liposome such that liposomes are  
24 spontaneously formed. A biologically active compound is also added prior to,

1 simultaneously or subsequent to the water and is present in a sufficient amount  
2 such that a biologically effective dosage of the biologically active compound is  
3 associated with the liposome. According to example 1 in the two U.S. patents,  
4 the pre-liposome composition is prepared at 50°C to 60°C. The component (a)  
5 (for example, lecithin) is dissolved in the component (b) (for example, ethanol).  
6 The water portion is then added in two parts. The first part consists of an  
7 appropriate amount of the biologically active compound (for example, in a  
8 glucose solution), and an amount of distilled water in the second part is required  
9 to make up the final formulation. The pre-liposome compositions are  
10 equilibrated under N<sub>2</sub>. Following equilibration, the pre-liposome compositions  
11 are cooled to 25°C. The liposomes are then prepared by a two-stage addition of  
12 phosphate buffer vigorously hand-shaken such that an aqueous dispersion of  
13 liposome that consists of a biologically active compound is formed. However,  
14 the ethanol is contained in the aqueous dispersion of liposome in a specific  
15 amount.

16 Francis C. Szoka, Jr. in U.S. Pat. Nos. 5,077,057, 5,277,914 and  
17 5,549,910 discloses a method for preparing a lipid suspension of defined particle  
18 size encapsulating a useful compound that has poor water, alcohol or  
19 halogenated hydrocarbon solubility. The poorly-soluble compound and an  
20 encapsulating amount of a suitable lipid are dissolved in an aprotic solvent such  
21 as DMSO, optionally containing a solubilizing amount of a lower alcohol (for  
22 example, ethanol), and then extruded or injected into a stirred or mixed aqueous  
23 solution. The resulting liposomal suspension may be dialyzed or otherwise  
24 concentrated, if desired. The extrusion means may be a syringe, perforated plate

1 or tube or other appropriate device providing apertures of about 0.05 mm to  
2 about 5 mm. In example 2 of the three U.S. Patents, doxorubicin is dissolved in  
3 DMSO and added to an ethanol solution containing egg phosphatidylglycerol  
4 (EPG): egg phosphatidylcholine (EPC): cholesterol (Chol) (7:3:6). Liposomes  
5 are formed by injecting the lipid-doxorubicin mixture into an aqueous phase  
6 consisting of 140 mM NaCl-10 mM Tris-HCl, pH 4.0, at 30°C. The liposome  
7 suspension is dialyzed and the liposome-encapsulated doxorubicin is separated  
8 from the nonencapsulated material by column chromatography. The resulting  
9 liposome particle diameter is 227 nm, and 41.2% of the doxorubicin is  
10 encapsulated in the liposome particles.

11 EP-A-560138 discloses a freeze-dried liposomal preparation containing  
12 a dihydro-pyridine compound that has a hydrophobic and low solubility  
13 character. U.S. Pat. No. 5,653,998 (R.O.C Pat. No. 359616) discloses that using  
14 short chain fatty acid (containing not greater than 10 carbon atoms) having a  
15 specific chemical formula or a salt of the fatty acid as a stabilizer causes the  
16 active compound having a hydrophobic and low solubility character to be  
17 encapsulated in the phospholipid membrane. The liposome can be a  
18 pharmaceutical preparation provided for parenteral delivery.

19 Martin C. Woodle et. al. in U.S. Pat. No. 5,013,556 disclose a liposome  
20 composition comprising liposomes composed of vesicle-forming lipids and  
21 between 1-20 mole percent of an amphipathic lipid derivatized with a  
22 polyethyleneglycol. One preferred amphipathic lipid is a phospholipid, such as  
23 phosphatidylethanolamine, derivatized with polyethyleneglycol. The liposomes  
24 can enhance circulation time in the bloodstream.

1           In example 10 of Francis J. Martin et. al. U.S. Pat. No. 5,213,804, a  
2   doxorubicin liposome is produced by using the liposome disclosed in U.S. Pat.  
3   No. 5,013,556. The selected lipid compositions in a selected mole ratio are  
4   dissolved in chloroform. Alpha-tocopherol ( $\alpha$ -TC) in free base form is added in  
5   chloroform:methanol (2:1) solution. The lipid solution is dried to a thin lipid film,  
6   then hydrated with a warm solution of 125 mM ammonium sulfate containing 1  
7   mM desferal. The lipid material is hydrated with 10 freeze/thaw cycles, using  
8   liquid nitrogen and a warm water bath. Liposome sizing is performed by  
9   extrusion. The extruded liposomes are then dialyzed against 5% glucose. A  
10   solution of doxorubicin is prepared and mixed with an equal volume of the  
11   dialyzed liposome preparation. The mixture is incubated at 60°C in a water bath  
12   with shaking. Doxorubicin is encapsulated in the liposome.

13           Yechezkel Barenholz et. al. in U.S. Pat. No. 5,192,549 disclose a loading  
14   procedure for efficient loading of amphiphatic drugs into liposomes. The loading  
15   of the amphiphatic drug into liposomes is dependent on  $\text{NH}_4^+$  gradients between  
16   the internal and external aqueous phases of the liposome vesicles, in which  
17   gradients are created by forming liposomes in the ammonium solution, by  
18   subsequent ammonium removal from or dilution in the external aqueous phase  
19   of the liposomes. The liposomes create an outflow of neutral ammonia based on  
20   the created ammonium gradient, from internal to external aqueous phase, thus  
21   creating an active reverse, from outside to inside, pH gradient by accumulation  
22   of protons left behind by ammonia in the internal aqueous phase. An influx of  
23   deprotonated amphiphatic drug to liposomes based on the pH gradient replaces  
24   the departed ammonium.

1           The conventional methods for producing liposome have several  
2   disadvantages. Some methods use chloroform or other toxic organic solvents to  
3   dissolve the lipid so the method must remove the toxic organic solvents. Some  
4   methods have limiting in solvent injection so the methods can not operate in  
5   aseptic manipulation. The uniformity and the quality of the liposome can be  
6   influence by rotation speed, injection rate, solvent ratio, injection needle size and  
7   so on. The extrusion process in some methods can make the pressure over 150 to  
8   250 psi, and the extrusion rate is very low (about 0.1 to 0.2 L/min). These  
9   disadvantages of the conventional methods will cause the manufacturing process  
10   to be time-consuming and prevent liposome from being manufactured in aseptic  
11   manipulation. The conventional methods cannot produce liposome in large  
12   scale.

### 13   SUMMARY OF THE INVENTION

14           The main objective of the present invention is to provide a process for  
15   producing liposome suspension in large scale and preventing the necessity to  
16   remove toxic organic solvent.

17           To achieve the objective, a process for producing liposome suspension in  
18   accordance with the present invention comprises: (a) providing a pre-mixture to  
19   an alcohol solvent, (b) mixing the pre-mixture with an aqueous ammonium  
20   sulfate solution to form a mixture, (c) subjecting the mixture to a pore-extrusion  
21   treatment to form a pre-liposome suspension and (d) dialyzing the pre-liposome  
22   suspension with 5% to 15% sucrose aqueous solution.

23           In step (a), the pre-mixture comprises

24                   (i) a phospholipid compound comprising 40%-70% of the pre-

1 mixture and selected from the group consisting of lecithin, phosphatidylcholine  
2 (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG),  
3 phosphatidylinositol, sphingomyelin (SM), phosphatidic acids, a di(C<sub>12</sub>-C<sub>18</sub>)acyl  
4 derivative of any of the foregoing and a combination of any of the foregoing;

5 (ii) a cholesterol comprising 10%-30% of the pre-mixture; and

6 (iii) a polyethyleneglycol (PEG)-derived compound comprising  
7 15%-30% of the pre-mixture and selected from the group consisting of PEG-PE,  
8 methoxy- polyethyleneglycol (mPEG)-PE, a di(C<sub>12</sub>-C<sub>18</sub>)acyl derivative of either  
9 of the foregoing and a combination of any of the foregoing;

10 wherein the mole ratio of the alcohol solvent to the total amount of  
11 the compounds (i), (ii) and (iii) is greater than 5:1.

12 In step (b), the pre-mixture obtained in step (a) is mixed with an aqueous  
13 ammonium sulfate solution to form a mixture and the ratio of the amount of the  
14 pre-mixture obtained in step (a) to the aqueous ammonium sulfate solution is  
15 1:2~10 (v/v).

16 In step (c), the mixture obtained in step (b) is subjected to a pore-  
17 extrusion treatment and formed a pre-liposome suspension.

18 In step (d), the pre-liposome suspension obtained in step (c) is dialyzing  
19 with a 5% to 15% sucrose aqueous solution such that a liposome suspension  
20 contained liposome particles suspended therein is obtained.

21 The liposome suspension produced by the present invention can use to  
22 encapsulate an amphipathic drug. The other objective of the present invention is  
23 to provide a process for producing a liposome-encapsulated drug comprising:

24 mixing a selected drug and a liposome suspension produced by the



1 foregoing process to produce a liposome-encapsulated drug contained the  
2 selected drug in the liposome particles in the liposome suspension. The selected  
3 drug is selected from the group consisting of an anthracycline antibiotic and a  
4 camptothecin anti-tumor drug. Preferably, the selected drug is selected from the  
5 group consisting of doxorubicin, daunorubicin, irinotecan and vinorelbine. More  
6 preferably, the selected drug is doxorubicin.

7 Further benefits and advantages of the present invention will become  
8 apparent after a careful reading of the detailed description with appropriate  
9 reference to the accompanying drawings.

#### 10 DETAILED DESCRIPTION OF THE INVENTION

11 A process for producing liposome suspension in accordance with the  
12 present invention comprises: (a) providing a pre-mixture to an alcohol solvent,  
13 (b) mixing the pre-mixture with an aqueous ammonium sulfate solution to form a  
14 mixture, (c) subjecting the mixture to a pore-extrusion treatment to form a pre-  
15 liposome suspension and (d) dialyzing the pre-liposome suspension with a 5% to  
16 15% sucrose aqueous solution.

17 In step (a), the pre-mixture comprises

18 (i) a phospholipid compound comprising 40%-70% of the pre-  
19 mixture and selected from the group consisting of lecithin, phosphatidylcholine  
20 (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG),  
21 phosphatidylinositol, sphingomyelin (SM), phosphatidic acids, a di(C12-  
22 C18)acyl derivative thereof and a combination of the foregoing;

23 (ii) a cholesterol comprising 10%-30% of the pre-mixture; and

24 (iii) a polyethyleneglycol (PEG)-derived compound comprising

1 15%-30% of the pre-mixture and selected from the group consisting of PEG-PE,  
2 methoxy- polyethyleneglycol (mPEG)-PE, a di(C<sub>12</sub>-C<sub>18</sub>)acyl derivative of any of  
3 the foregoing and a combination of the foregoing;

4                   Wherein the mole ratio of the alcohol solvent to the total amount of  
5 compounds (i), (ii) and (iii) is more than 5:1.

6           In step (b), the pre-mixture obtained in step (a) is mixed with an aqueous  
7 ammonium sulfate solution to form a mixture and the ratio of the amount of the  
8 pre-mixture obtained in step (a) to the aqueous ammonium sulfate solution is  
9 1:2~10 (v/v).

10           In step (c), the mixture obtained in step (b) is subjected to a pore-  
11 extrusion treatment and forms a pre-liposome suspension.

12           In step (d), the pre-liposome suspension obtained in step (c) is dialyzed  
13 with a 5% to 15% sucrose aqueous solution such that a liposome suspension  
14 containing suspended liposome particles is obtained.

15           The alcohol solvent used in step (a) in the process is a non-toxic alcohol  
16 solvent and is water-soluble. Preferably, the alcohol solvent is selected from the  
17 group consisting of fatty alcohol, glycol, methanol, ethanol, i-propanol, ethylene  
18 glycol, propylene glycol and a combination of the foregoing alcohol solvents. In  
19 a preferred embodiment, the alcohol solvent used in step (a) is ethanol.

20           Preferably, the compound (i) used in step (a) is selected from the group  
21 consisting of PC, dilauroyl PC, dimyristoyl PC, dipalmitoyl PC, distearoyl  
22 phosphatidylcholine (DSPC), dioleoyl PC, dilinoleoyl PC, 1-palmitoyl-2-oleoyl  
23 PC and a combination of the foregoing compounds. In a preferred embodiment,  
24 the compound (i) used in step (a) is DSPC.

1 Preferably, the compound (iii) used in step (a) is selected from the group  
2 consisting of PEG-2000-PE, PEG-3000-PE, PEG-4000-PE, PEG-5000-PE,  
3 mPEG-2000-PE, mPEG-3000-PE, mPEG-4000-PE, mPEG-5000-PE, a di(C<sub>12</sub>-  
4 C<sub>18</sub>)acyl derivative of the foregoing compounds and a combination of any of the  
5 foregoing compounds.

6 More preferably, the compound (iii) used in step (a) is selected from the  
7 group consisting of PEG-2000- distearoyl phosphatidylethanolamine (DSPE),  
8 PEG-3000-DSPE, PEG-4000-DSPE, PEG-5000-DSPE and a di(C<sub>12</sub>-C<sub>18</sub>)acyl  
9 derivative of the foregoing.

10 The di(C<sub>12</sub>-C<sub>18</sub>)acyl derivative of compound (iii) used in step (a) can be  
11 but is not limited to 1,2-diacyl-SN-glycero-3-phosphatidyl ethanolamine-N-  
12 [methoxy(polyethylene glycol)-2000] and 1,2-diacyl-SN-glycero-3-  
13 phosphatidyl ethanolamine-N-[methoxy(polyethylene glycol)-3000]. The acyl is  
14 myristoyl, palmitoyl, stearoyl or oleoyl. In a preferred embodiment, the  
15 compound (iii) used in step (a) is PEG-2000-DSPE.

16 Preferably, the ratio of the amount of the alcohol solvent and the total  
17 amount of the compounds (i), (ii) and (iii) is 7~10:1 (w/v). Preferably, the ratio  
18 of the amount of the pre-mixture obtained in step (a) to the aqueous ammonium  
19 sulfate solution is 1: 4~8 (v/v).

20 In a preferred embodiment, DSPC, cholesterol and PEG-2000-DSPE as  
21 components of liposome particles are dissolved in ethanol.

22 In a preferred embodiment, step (a) is carried out at 45°C to 70°C.  
23 Preferably, the step (a) is carried out at 55°C to 65°C. More preferably, the step  
24 (a) is carried out at 60°C.

1           In a preferred embodiment, DSPC, cholesterol and PEG-2000-DSPE are  
2 dissolved in ethanol and mixed well in a water bath at 60°C.

3           In the step (b) of the process, the lipid/organic solvent mixture is not like  
4 the convention process that uses a syringe to inject an small amounts of aqueous  
5 solution several times. The present process can directly add the aqueous  
6 ammonium sulfate solution to the pre-mixture obtained in step (a) or directly add  
7 the pre-mixture obtained in step (a) to the aqueous ammonium sulfate solution.

8           Preferably, step (b) is carried out at 45°C to 70°C. More preferably, the  
9 step (b) is carried out at 55°C to 65°C. Most preferably, the step (b) is carried out  
10 at 60°C. At this operation temperature, the lipid bilayer membrane structure of  
11 the liposome particle in the mixture is loose. The liposome particle will  
12 encapsulate small amounts of aqueous ammonium sulfate solution.

13           Preferably, the equivalent weight of the aqueous ammonium sulfate  
14 solution in step (b) is 0.2N to 0.8N. More preferably, the equivalent weight of the  
15 aqueous ammonium sulfate solution in step (b) is 0.4N to 0.6N.

16           Preferably, the pore-extrusion treatment in step (c) passes the mixture  
17 obtained in step (b) through a device with apertures of 0.05μm to 0.45μm.

18           A device to perform the pore-extrusion treatment in step (c) in  
19 accordance with the present invention may be a syringe providing apertures of  
20 about 0.05 μm to 0.45μm, a filter containing a ceramic filtration membrane or a  
21 polycarbonate filtration membrane or a plate or tube with apertures.

22           Preferably, the pore-extrusion treatment in step (c) has two steps and  
23 first passes the mixture obtained in step (b) through a filter having large  
24 apertures and then through a filter having small apertures. In a preferred

embodiment, the pore-extrusion treatment in step (c) passes the mixture obtained in step (b) through a filter having apertures of 0.1  $\mu\text{m}$  and then a filter having apertures of 0.05  $\mu\text{m}$  at 60°C.

Preferably, step (d) is carried out at room temperature. Under these conditions, the lipid bilayer of the liposome particle will become dense, and the aqueous ammonium sulfate solution will not escape from the inside of the liposome particle to the sucrose solution during dialyzation.

The liposome suspension obtained in step (d) can be using immediately or can be stored at a low temperature (for example, 5°C), or the obtained liposome suspension is further lyophilized and stored at a low temperature (for example, 5°C).

Compared with the conventional process, the process for producing liposome suspension in accordance with the present invention can be performed at a low pressure (about 40 to 140 psi), and the yield increases (about 2 to 10 L/minute). The present invention can provide a quick and economic process for producing liposome suspension in large quantities.

The liposome suspension produced by the present invention can be used in pharmaceuticals and cosmetics, more particularly for encapsulating a selected drug that can be but is not limit to anthracycline antibiotics and camptothecin anti-tumor drugs.

Thus, the present invention also provides a process for producing a liposome-encapsulated drug comprising:

mixing a selected drug and a liposome suspension produced by the foregoing process to produce a liposome-encapsulated drug containing the

1 selected drug in the liposome particles in the liposome suspension.

2 Preferably, the selected drug is selected from the group consisting of  
3 doxorubicin, daunorubicin, irinotecan and vinorelbine.

4 Preferably, the selected drug and liposome suspension are mixed at 45°C  
5 to 70°C. More preferably, the selected drug and liposome suspension are mixed  
6 at 55°C to 65°C. Most preferably, the selected drug and liposome suspension are  
7 mixed at 60°C. At this temperature, the lipid bilayer of the liposome particle is  
8 loose and allows the selected drug to enter the liposome particles and bind with  
9 the ammonium sulfate. The temperature is decreased to room temperature, and  
10 the lipid bilayer of the liposome particle becomes more dense such that the  
11 selected drug is stably encapsulated in the liposome particles in the liposome  
12 suspension.

13 Preferably, a pharmaceutical acceptable additive is used when a drug is  
14 encapsulated. For example, the pharmaceutical acceptable additive is a  
15 cryoprotector, an antioxidant formed stabilizer, a pH-regulator, a dispersing  
16 agent or a combination of the foregoing. For example the cryoprotector may be  
17 polyol (glycerol), monosaccharide (glucose), disaccharide (sucrose, lactose,  
18 trehalose), a protein or an amino acid (histidine). The antioxidant formed  
19 stabilizer may be butylated hydroxyanisole (BHA), butylated hydroxytoluene  
20 (BHT),  $\alpha$ -tocopherol or a salt of  $\alpha$ -tocopherol, ascorbic acid or a salt or an ester  
21 of ascorbic acid, preferably is ascorbic acid and a salt thereof. The pH-regulator  
22 may be a buffer, an acid or a base (for example ascorbic acid and NaOH). The  
23 dispersing agent may be glycerol, mannitol or glucose.

24 In a preferred embodiment, the selected drug is doxorubicin that is

1 encapsulated in the liposome suspension produced by the present invention and  
2 histidine as the stabilizer.

3 All of the documents or publications recited in the text are incorporated  
4 herein by reference.

5 Further details of this invention are illustrated in the following  
6 examples.

7

## 8 Materials

### 9 A. Aqueous ammonium sulfate solution:

10 158 g ammonium sulfate (Showa Co., Japan) was added to injection  
11 water, and the volume was adjusted to 6 L. The solution was filtered with  
12 Posydine (Whatman Inc., Germany) thin membrane (142mm, 0.22 $\mu$ m) and  
13 stored at 6°C. Before using, the ammonium sulfate aqueous solution was  
14 adjusted at 60°C.

### 15 B. 9% (w/w) sucrose solution:

16 4500 g sucrose was dissolved in a suitable amount of injection water and  
17 diluted to 50L, and then filtered with Posydine thin membrane (142 mm,  
18 0.22 $\mu$ m).

### 19 C. Histidine-sucrose solution:

20 12.6 g histidine monohydrate (Ajinomoto Co., Japan) was dissolved in  
21 90 mL 9% sucrose solution, and 1N NaOH was used to adjust the range of the pH  
22 from 6.2 to 6.6. 9% sucrose solution was added until the volume was 300 mL and  
23 mixed well. The solution was filtered with Posydine thin membrane (47mm,  
24 0.22 $\mu$ m).

1

## 2 Example 1 Preparation of liposome suspension

3 16.8 g of PEG-2000-DSPE (Genzyme Co., America), 27.4 g of  
4 cholesterol (NOF Co., Japan) and 38.2 g of DSPC (NOF Co., Japan) were added  
5 to 600 ml of ethanol in a glass container. The mixture was stirred at 60°C and  
6 mixed well. While continuously stirring the mixture and maintaining the mixture  
7 at 60°C, 4L of the aqueous ammonium sulfate solution was directly added to the  
8 mixture. At the temperature, the ethanol was almost evaporated. Then the  
9 mixture was subjected to a pore-extrusion treatment using a 1.5L of filter  
10 (Advantec Toyo Kaisha, Ltd., Japan), and the pore-extrusion treatment  
11 comprised

12 (1) a filter having a first filtration membrane (142 mm, 0.1  $\mu\text{m}$ ) and  
13 filtering the mixture 10 times; and

14 (2) changing to a second filtration membrane (142 mm, 0.05  $\mu\text{m}$ ) and  
15 filtering the mixture more 10 times.

16 The extrusion pressure was controlled at 3 to 10  $\text{kg}/\text{cm}^2$  and the flow rate  
17 was about 2 to 10 L/min. 4500 mL of filtration solution was collected and then  
18 dialyzed with 30 L 9% (w/w) sucrose solution previously prepared in a 30KD  
19 hollow fiber (A/G Technology, UFP-30-C-6A, 30,000 NM, 4800  $\text{cm}^2$ ). The  
20 remnant ethanol was all removed by dialyzing. The volume of the collected  
21 solution was about 3000 mL, and the collected solution was a liposome  
22 suspension that did not contain ethanol.

## 23 **Product quality analysis**

24 A sample of the liposome suspension produced in example 1 was



analyzed with a GC analyzer (Varian, Inc., America). The results showed that the sample of the liposome suspension contained no ethanol. A particle size analysis was performed on another sample with a particle size analyzer (Beckman coulter, Inc.). The results showed the average particle size in the liposome suspension in the sample to be 72.9 nm.

#### Example 2: Preparation of liposome encapsulated doxorubicin

3000 mL of liposome suspension produced in example 1 was added to a glass container containing 8000 mg doxorubicin HCl (red powder), and 200 mL histidine-sucrose solution previously prepared was continuously added. A mixture was formed and put in a 60°C water bath and stirred for 30 minutes. The mixture was then cooled to about 35°C, diluted with 9% sucrose solution to 4 L and mixed well. A liposome-encapsulated doxorubicin was produced. The product was further packaged in sterile glass vials and manufactured an injection preparation containing 2.0 mg doxorubicin HCl/mL.

#### **Product quality analysis**

The color and luster of the liposome-encapsulated doxorubicin produced in example 2 was observed. The color of the injection preparation packaged in the sterile glass vials was reddish orange to red. A sample was analyzed with an HPLC analyzer (Waters co., America) and compared with the standard. The results showed that the retention time and elution profile of the doxorubicin HCl in the sample were the same as that of the standard.

The liposome-encapsulated doxorubicin produced in example 2 was analyzed to determine the amount of doxorubicin encapsulated. 99.61% of the

1 doxorubicin was encapsulated in the liposome. A sample was analyzed to  
2 determine the particle size with a particle size analyzer (Beckman coulter, Inc.),  
3 and the results showed that the particle size of the liposome in the sample was  
4 91.0 nm.

5 The liposome-encapsulated doxorubicin produced in example 2 was  
6 stored at 2 to 8°C for 30 months. Testing of a stored sample showed that the  
7 stored sample was stable (table 1), and the particle size of the liposome had not  
8 apparently changed.

9 Table 1: Stability of the Injection Preparation of the Liposome-  
10 encapsulated Doxorubicin in Example 2

Time	Concentration (mg/mL)	Particle size (nm)
	5°C±3°C	5°C±3°C
Beginning	1.99±0.02	91.0±22.2
1 <sup>st</sup> month	1.97±0.05	95.2±24.1
12 <sup>th</sup> month	1.95±0.07	90.4±18.3
24 <sup>th</sup> month	1.96±0.03	96.3±23.1
30 <sup>th</sup> month	1.98±0.01	93.3±26.6

11 Although the invention has been explained in relation to its preferred  
12 embodiment, many other possible modifications and variations can be made  
13 without departing from the spirit and scope of the invention as hereinafter  
14 claimed.